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The effect of applied arachidonic acid on the formation of prostaglandins in plantlets from excised apices of the short-day plant, *Pharbitis nil*

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The effect of arachidonic acid (AA) application to excised apices of *Pharbitis nil* and the subsequent production of prostaglandins (PGs) were examined. The detection of the PGs was done by means of a radioimmunoassay (RIA) and tritium labelling techniques. Both PGE_2 and $PGF_{2\alpha}$ were detected and quantified by RIA after AA application. This suggests the synthesis of PGs from AA. These results were confirmed using tritium labelled AA. This experiment was done in connection with the fact that *Pharbitis nil* is a short-day plant. Arachidonic acid, a possible precursor of PGs, possibly promotes flowering of this plant. Arachidonic acid might be converted by cyclo-oxygenases to PGs which are involved in flowering.

Introduction

Arachidonic acid (20:4) is a direct precursor of prostaglandins (PGs) in mammalian systems (Hinman 1972). Prostaglandins were discovered in plants in the early seventies (Attrep *et al*. 1973) and have since been found in many different plants, where they have different physiological effects (Panossian 1987, Yurin 1991, Groenewald and Van der Westhuizen 1997).

The aim of this study was to ascertain whether applied arachidonic acid (AA) could be converted by excised apices of *Pharbitis nil* to PGs (PGE_2 and $PGF_{2\alpha}$) as in mammals. It has been shown by various researchers that labelled AA could be converted to PGE_2 and $PGF_{2\alpha}$ in plant homogenates from maize (Forster *et al*. 1984), different *Allium* species (Ali *et al*. 1990) and *Aloe vera* (Afzal *et al.* 1991).

It has long been thought that PGs possibly play a role in the flowering of *P. nil*, a short-day plant. Evidence for this comes from studies of inhibitors of PG biosynthesis on flowering of intact plants and plantlets from excised apices of *P. nil* (Groenewald and Visser 1974, 1978, Groenewald and Van der Westhuizen 1998). The various inhibitors inhibited flowering to a greater or lesser extent.

Previous studies revealed that application of AA and PGE_1 to apices of *P. nil* under inductive conditions (short days) hastened the floral transition of plantlets by approximately 60% (Groenewald and Visser 1978). This implies that the applied AA and PG are possibly involved in flowering of *P. nil* plantlets.

Materials and Methods

Preparation of apices

Seeds of *Pharbitis nil* Chois. cv. Violet (obtained from Muratane Co. Ltd, Kyoto, Japan) were suspended in concentrated H_2SO_4 for 45min to soften the testa and then rinsed with distilled H_2O to remove excess acid. The seeds were next treated for 10min with 70% ethanol (EtOH), followed by 60min in 10% $Ca(OCl)_2$. The seeds were then washed in sterile distilled H_2O and treated for 10min with a 1:1 (v/v) mixture of 0.1% $HgCl_2$ and 0.1% sodium lauryl sulphate, before washing with three changes of sterile distilled H_2O. The sterilised seed were aseptically transferred to 250ml Erlenmeyer flasks containing sterilised moistened cotton wool. The seeds were allowed to germinate on the cotton wool in the flasks under continuous light (345µE m^{-2} s^{-1}) in a constant temperature growth chamber of which the air temperature was 27°C. After three days, the stems of the seedlings were about 4–5cm long and they were then aseptically transferred to sterile petri dishes. The cotyledons were removed using a sterile scalpel and the hypocotyl was severed 5–10mm below the stem apex. The stem apices thus excised were placed with the cut surface pressed onto a suitable agar nutrient medium contained in a test tube (5ml medium in a 23mm x 195mm tube). The nutrient medium used was the mineral salt mixture of Linsmaier and Skoog (1965) supplemented with 3% sucrose, 0.8% agar, 1g l^{-1} casein hydrolysate, 0.4mg l^{-1} thiamine-HCl, 100mg l^{-1} myo-inositol and 1mg l^{-1} kinetin. The pH was adjusted to 5.8 prior to autoclaving. Using this medium, relatively large flowers (c. 3cm long) were obtained under inductive photoperiods.

The shoot apices were usually kept on the nutrient agar medium lacking the test compound (AA) for three days to ensure that the apices were not infected with fungus. The apices were kept at 25°C and under long day conditions (16h light and 8h darkness). At the end of this period a small segment (c. 5mm) was excised from the base (cut end) of the hypocotyl of each explant and the apices were transferred to agar nutrient medium containing the test compound (AA).

Radio-immunoassay

A 10^{-4}M solution of AA was prepared by dissolving every 1mg of AA in 0.1ml of 95% EtOH, to which was added 0.9ml of an aqueous solution of 0.2mg ml^{-1} Na_2CO_3. The pH was adjusted to between 6 and 7.5 by measurement using pHydrion paper. After filter-sterilisation through a Millipore filter (0.45µm pore size), the stock was added to the autoclaved agar nutrient medium (0.2ml per 5ml of agar medium) after it reached a temperature of 40–50°C and mixed thoroughly before the mixture was allowed to solidify.

Eighty test tubes containing excised apices were employed in the experiment. A similar number of apices in nutrient medium, without AA, were used as controls. The test tubes containing the apices were placed in a temperature controlled growth chamber at 25°C under short day conditions (8h light and 16h darkness) for eight days.

At the end of this period the plantlets which developed from apices were taken out of the agar and extracted for PGs. The methods of Lee *et al*. (1967) and Krüger *et al*. (1990) were used for the extraction and partial purification of the PGs, respectively. The PGs were further purified and chromatographed (Groenewald *et al*. 1994) in preparation for their quantification using radio-immunoassay (RIA). The two prostaglandins (PGE_2 and $PGF_{2\alpha}$) were detected by using two different RIA kits (Clinical Assays, Cambridge, Massachusetts, USA), based on monoclonal antibodies specific for the two different PGs. Each RIA was performed in duplicate and the results were essentially similar.

The assay procedure included the preparation of two standard curves, one each for PGE_2 and $PGF_{2\alpha}$, in which radioactive PG (from kit) and non-radioactive PG (from sample to be assayed) compete for a fixed amount of PG antibody. The standard curves were then used to determine the PG content of the assay samples from the percentage binding of radioactive PG to the antibody.

Tritium labelling study

In another study, tritium labelled AA was applied to aseptically excised apices of *P. nil*. The total radioactivity of the AA was 92.5 x 10^5Bq and it was received as a solution in 0.25ml hexane. The specific activity was 266.4 x 10^{10}Bq mol^{-1}. The solution was evaporated to dryness under reduced pressure and the radioactive AA was dissolved in 0.5ml 96% EtOH. To this was added 5mg non-radioactive AA and 4.5ml Na_2CO_3-solution (0.2mg $Na_2CO_3ml^{-1}$ H_2O). The pH of the final solution was approximately 7 as measured with pHydrion paper and the volume of the solution was 5ml. The solution was sterilised by means of a Millipore filter (pore size 0.45µm). Aliquots of 0.02ml of the filter sterilised AA solution (containing 3.7 x 10^4Bq of radioactive material) were added to 2ml agar nutrient medium contained in test tubes while the agar was still in liquid form. The composition of the agar medium was the same as that used for the RIA experiment. Seventy tubes containing the labelled AA were used and each received a single aseptic *P. nil* apex. The test tubes with excised apices were placed in a temperature controlled growth chamber at 27°C under short day conditions (8h light and 16h darkness) for eight days. At the end of this period the apices were taken out of the agar and extracted for PGs according to the methods described for the RIA experiment. During the final extraction procedure the PGs were extracted with chloroform. This fraction was evaporated to 0.5ml and this was used for chromatography. Silica gel 20cm x 20cm thin layer plates on aluminium (Merck) were used for one dimensional chromatography and two solvent systems were used, namely, ethyl acetate–methanol–H_2O (160:3:100 v/v/v, organic phase) and benzene–dioxane–acetic acid (20:20:1 v/v/v). At the conclusion of the chromatographic run, the plates were air dried and the region where the labelled extract chromatographed was divided into cm^2 sections along the length of the developed plate. The ethyl acetate-MeOH-H_2O plate was divided into 15cm^2 sections since the solvent front was 15cm from the origin. The other plate (bezene–dioxane–acetic acid) was divided into 10cm^2 sections (solvent front was 10cm from origin). Authentic PGE_2 and $PGF_{2\alpha}$, as well as their putative breakdown products PGA_2 and PGB_2 were simultaneously chromatographed alongside the labelled extract. The exact positions (Rfs) of the authentic PGs were detected by a non-destructive treatment of the plates with iodine vapours.

The cm^2 sections were separately cut out and were counted separately using a Tricarb scintillation counter.

Results and Discussion

The RIA data (Table 1) provide convincing evidence that *Pharbitis nil* can synthesise PGE_2 and $PGF_{2\alpha}$ from AA. The average amount of PG per explant was 4.7ng and 5.0ng for PGE_2 and $PGF_{2\alpha}$ respectively. Determinations of PG levels in other plants (Groenewald *et al.* 1994) have indicated that concentrations of $PGF_{2\alpha}$ vary from 0.136ng to 28ng g^{-1} dry weight, depending on the type of plant. It is also interesting in this regard that Levin *et al.* (1990) detected AA and differ-

Table 1: Prostaglandin content of plantlets as determined by radio-immunoassay

Treatment	Prostaglandin content	
	PGE_2 (ng per 80 explants)	$PGF_{2\alpha}$ (ng per 80 explants)
+ Arachidonic acid	333.3	400.0
– Arachidonic acid (control)	Trace	Trace

ent PGs only in buds and not in leaves of poplar (*Populus balsamifera*). In March, the AA and PG contents of trees that grew in Siberia was 2.9µg and 9.5µg g^{-1} dry weight respectively, whereas in April it was 52.3µg and 71.0µg g^{-1} dry weight respectively. These increases correspond with the start of summer and are consistent with the role of AA as the precursor of PGs in buds. In contrast with these Russian researchers who detected microgram quantities of these compounds, other workers who studied different species detected only nanogram quantities when expressed per gram dry weight.

The results shown in Table 2 indicate that the count percentages peaked in fractions 1, 5–8 and 10. Fraction 1 is the application site of the extract. Fraction 5 ($PGF_{2\alpha}$) shows 11% of total counts and fraction 7 (PGE_2) 32% of total counts. The relatively high percentage counts in fractions 6 and 8 likely results from overlapping and tailing effects during the chromatography. The percentage counts of PGA_2 and PGB_2, (Fraction 10) which did not separate in this solvent system, is relatively low. The relative high percentage count in fraction 1 may be due to residues of the applied sample. The data in this case suggest that AA has been converted to PGE_2 and $PGF_{2\alpha}$ by plantlets of *P. nil.* Because of the unsatisfactory separation of fractions 5, 6, 7 and 8 the chromatography was repeated in a different solvent system (Table 3).

According to Table 3, peak percentage counts were found in fractions 1, 4, 8, 10, 11 and 12. These have Rf-values corresponding to the application site of the extract, $PGF_{2\alpha}$ PGE_2, PGA_2, PGB_2 and AA respectively. PGA_2 and PGB_2 could be present as such or as breakdown products of PGE_2 and $PGF_{2\alpha}$. Fraction 12 is probably unmetabolised residue of AA. Fraction 1 is at the application site that contained residues of the applied sample.

Collectively, all of the data are consistent with the conversion of AA to PGs in excised apices of *P. nil* and imply that an enzyme system similar or identical to the mammalian cyclo-oxygenase pathway is active in the explants. In mammals, certain PGs and other eicosanoids (such as prostacyclins, and thromboxanes) are formed from the phospholipid fraction in cell membranes. Phospholipid is hydrolysed by phospholipase A_2 to form AA, with the subsequent formation of PGs occurring as follows: AA $\rightarrow$ PGG_2 $\rightarrow$ PGH_2 $\rightarrow$ PGE_2 and $PGF_{2\alpha}$. The enzyme prostaglandin H_2 synthetase catalyses the first and second steps, which involve cyclo-oxygenase and peroxidase activities respectively. Finally PGH_2 is converted to PGE_2 and $PGF_{2\alpha}$, which are the two most abundant PGs in mammals (Voet and Voet 1995).

Definitive evidence of a plant cyclo-oxygenase is lacking, although there is circumstantial evidence of the existence of this enzyme in plants (Ali *et al.* 1990, Afzal *et al.* 1991).

Table 2: The separation of radioactive fractions of a plant extract on a silica gel thin layer plate, developed in benzene–dioxane–acetic acid. Authentic prostaglandins were co-chromatographed and counts are given as percentages of total counts (1 227cpm)

Fractions (cm^2)	Fraction of total counts (%)	Identified compounds in fractions	Rf-values
1	9		
2	6		
3	6		
4	7		
5	11	$PGF_{2\alpha}$	0.50
6	15		
7	32	PGE_2	0.70
8	11		
9	4		
10	8	PGA_2, PGB_2	1.0

Table 3: The separation of radioactive fractions of a plant extract on a silica gel thin layer plate, developed in ethyl acetate–MeOH–H_20. Authentic prostaglandins were co-chromatographed and counts are given as percentages of total counts (2 046cpm)

Fractions (cm^2)	Fraction of total counts (%)	Identified compounds in fractions	Rf-values
1	11		
2	3		
3	2		
4	7	$PGF_{2\alpha}$	0.27
5	3		
6	4		
7	5		
8	11	PGE_2	0.53
9	4		
10	13	PGA_2	0.67
11	15	PGB_2	0.73
12	12	Arachidonic acid	0.80
13	5		
14	3		
15	3		

Furthermore, Sanz *et al.* (1998) reported a pathogen-induced oxygenase (piox) from tobacco that bears homology with animal cyclo-oxygenases and catalyses the oxygenation of polyunsaturated fatty acids. Induction of piox expression in leaf tissue by inoculation with bacteria as well as in response to various cellular signals implicate its participation in stress-related phenomena such as the oxidative burst that occurs during cell death. Conceivably, cyclo-oxygenase expression might precede flowering, which can also be considered to be a stressful event (Leopold 1964). In our experiments, the plantlets were grown under inductive conditions (short days) to induce flowering and this could have induced the enzyme system necessary for the conversion of AA to PGs, since this could have been the start of a stressful situation.

To summarise, the data from both RIA and tritium labelling experiments indicate for the first time that plantlets of *P. nil* can convert AA to PGE_2 and $PGF_{2\alpha}$ and imply that this species may possess an enzyme system such as the cyclo-oxygenase system responsible for PG synthesis in mammals.

References

Afzal M, Ali M, Hassan RAH, Sweedan N, Dhami MSI (1991) Identification of some prostanoids in *Aloe vera* extracts. Planta Medica **57**: 38–40

Ali M, Afzal M, Hassan RAH, Farid A, Burka JF (1990) Comparative study of the *in vitro* synthesis of prostaglandins and thromboxanes in plants belonging to Liliaceae family. General Pharmacology **21**: 273–276

Attrep KA, Mariani JM, Attrep M (1973) Search for prostaglandin A_1 in onion. Lipids **8**: 484–486

Forster T, Szarvas T, Tanacs B (1984) Plant physiological effects of $PGF_{2\alpha}$ and prostaglandin synthesis in corn-leaf. Acta Biochimica et Biophysica Academiae Scientiarum Hungaricae **19**: 42

Groenewald EG, Van der Westhuizen AJ (1997) Prostaglandins and related substances in plants. The Botanical Review **63**: 199–220

Groenewald EG, Van der Westhuizen AJ (1998) The metabolism of inhibitor of flowering and prostaglandin biosynthesis, acetylsalicylic acid, in *Pharbitis nil* cotyledons. Biologia Plantarum **41**: 475–479

Groenewald EG, Van der Westhuizen AJ, Botes P (1994) Prostaglandin F_{2a} and g-linolenic acid in *Oenothera stricta*. Planta Medica **60**: 84–85

Groenewald EG, Visser JH (1974) The effect of certain inhibitors of prostaglandin biosynthesis on flowering of *Pharbitis nil*. Zeitschrift für Pflanzenphysiologie **71**: 67–70

Groenewald EG, Visser JH (1978) The effect of arachidonic acid, prostaglandins and inhibitors of prostaglandin synthetase, on the flowering of excised *Pharbitis nil* shoot apices under different photoperiods. Zeitschrift für Pflanzenphysiologie **88**: 423–429

Hinman JW (1972) Prostaglandins. Annual Review of Biochemistry **41**: 161–178

Krüger GHJ, Groenewald EG, De Wet H, Botes PJ (1990) The occurrence of prostaglandin $F_{2\alpha}$ in prokaryotic organisms. South African Journal of Botany **56**: 150–153

Lee JB, Crowshaw K, Takman BH, Attrep KA, Gougoutas JZ (1967) The identification of prostaglandins E_2, $F_{2\alpha}$ and A_2 from rabbit kidney medulla. Biochemical Journal **105**: 1251–1260

Leopold AC (1964) Plant Growth and Development. McGraw-Hill, New York, pp 197–199. ISBN 64–21071

Levin ED, Isaeva EV, Cherepanova VE (1990) Arachidonic acid and prostaglandins in buds of *Populus balsamifera*. Phytochemistry **29**: 2325–2326

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiologia Plantarum **18**: 100–127

Panossian AG (1987) A review of the occurrence of prostaglandins and prostaglandin-like compounds in plants. Prostaglandins **33**: 363–381

Sanz A, Moreno JI, Castresana C (1998) PIOX, a new pathogen-induced oxygenase with homology to animal cyclo-oxygenase. The Plant Cell **10**: 1523–1537

Voet D, Voet JG (1995) Biochemistry (2^{nd} edn). John Wiley and Sons, pp 706–708. ISBN 0–471–58651–X

Yurin VM (1991) Prostaglandins in plants. Soviet Plant Physiology **37**: 609–614

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